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(54) Title: IDENTIFICATION OF ALLELE SPECIFIC NUCLEIC ACID SEQUENCES BY HYBRIDIZATION WITH CROSSLINKABLE OLIGONUCLEOTIDE PROBES

(57) Abstract

Hybridization method for discriminating between complementarity and partial complementarity of DNA base sequences. Kinetic covalent entrapment of PCR-amplified target DNA is achieved through the use of crosslinkable probes. A crosslinking site is introduced into the target DNA via the PCR amplification process. Problems with renaturation of target DNA in the PCR process and in the hybridization reaction are minimized.

≈ M

Nested primers p 19.

Primers with x-linking agents

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M   Q1/70 B2B

IDENTIFICATION OF ALLELE SPECIFIC
NUCLEIC ACID SEQUENCES BY HYBRIDIZATION
WITH CROSSLINKABLE OLIGONUCLEOTIDE PROBES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to nucleic acid hybridization with crosslinkable oligonucleotide probes, and in particular, (1) a chemical synthesis route for the production of psoralen monoadducted oligonucleotide probes, (2) a method for the site-specific placement of a crosslinking site and/or a cross linking reagent during the enzymatic amplification of one or more segments of one or more nucleic acid targets, and (3) a hybridization method for discriminating between two or more nucleic acid base sequences in one or more nucleic acid targets that differ by a single base.

Description of the Prior Art

The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interactions is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane, Proc. Nat. Acad. Sci., U.S.A. 46, 453 (1960) and Doty et al., Proc. Nat. Acad. Sci., U.S.A. 46, 461 (1960) have been followed by the refinement of this process into an essential tool of modern biology.

Initial hybridization studies, such as those performed by Hayashi et al., Proc. Nat. Acad. Sci., U.S.A. 50, 664 (1963), were performed in solution. Further development led to the immobilization of the target DNA or RNA on solid supports. With the discovery of specific restriction endonucleases by Smith and Wilcox, J. Mol. Biol. 51, 379 (1970), it became possible to isolate discrete fragments of DNA.

Utilization of immobilization techniques, such as those described by Southern, J. Mol. Biol. 98, 503 (1975), in combination with restriction enzymes, has allowed for the identification by hybridization of single copy genes

5   among a mass of fractionated, genomic DNA.

In spite of the progress made in methodology, a number of problems have prevented the wide scale use of hybridization as a tool in human diagnostics. Among the more formidable problems are a) the inefficiency of

10   hybridization, b) the low concentration of specific target sequences in a mixture of genomic DNA, and c) the hybridization of only partially complementary probes and targets.

a.   Inefficient Hybridization

15   It is experimentally observed that only a fraction of the possible number of probe-target complexes are formed in a hybridization reaction. This is particularly true with short oligonucleotide probes (less than 100 bases in length). There are three

20   fundamental causes: 1) hybridization cannot occur because of secondary and tertiary structure interactions, 2) strands of DNA containing the target sequence have rehybridized (reannealed) to their complementary strand, and 3) some target molecules are

25   prevented from hybridization when they are used in hybridization formats that immobilize the target nucleic acids to a solid surface.

Even where the sequence of a probe is completely complementary to the sequence of the target, i.e. the

30   target's primary structure, the target sequence must be made accessible to the probe via rearrangements of higher-order structure. These higher-order structural rearrangements may concern either the secondary structure or tertiary structure of the molecule.

35   Secondary structure is determined by intramolecular

bonding.   In the case of DNA or RNA targets this consists of hybridization within a single, contiguous strand of bases (as opposed to hybridization between two different strands). Depending on the extent and

5   position of intramolecular bonding, the probe can be displaced from the target sequence, preventing hybridization.

Solution hybridization of oligonucleotide probes to denatured double-stranded DNA is further complicated by

10   the fact that the longer complementary target strands can renature or reanneal. Again, hybridized probe is displaced by this process. This results in a low yield of hybridization (low "coverage") relative to the starting concentrations of probe and target.

15   The immobilization of target nucleic acids to solid surfaces such as nylon or nitrocellulose is a common practice in molecular biology. Immobilization formats eliminate the reassociation problem that can occur between complementary strands of target molecules, but

20   not the problems associated with secondary structure effects. However, these mixed phase formats (i.e., Southern hybridization or dot blot hybridization) require time consuming fixation procedures. The hybridization reaction itself is kinetically much slower

25   than a solution phase hybridization reaction. Together, the fixation and hybridization procedures require a minimum of several hours to several days to perform. Additionally, the standard immobilization procedures are often inefficient and result in the attachment of many

30   of the target molecules to multiple positions on the solid surface, rendering them incapable of subsequent hybridization to probe molecules. Overall, these combined effects result in just a few percent of the initial target molecules being bound by probes in a

35   hybridization reaction.

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Yabusaki et al., U.S. Patent No. 4,559,303, describe a nucleic acid hybridization assay employing probes that can be covalently crosslinked to their targets. A crosslinking reagent is required as well as a crosslinking site in both the probe and target nucleic acid sequences. The presence of the crosslinking reagent on the probe, by itself, does not effect the kinetics of hybridization, the specificity of base pairing, or the stability of the probe-target complex. Covalently crosslinked probes do have, however, several unique advantages over conventional probes, the most important of which is the ability to use denaturing conditions to remove non-specifically bound probe. This capability allows for simpler hybridization formats and permits the use of crosslinkable probes at concentrations much higher than conventional probes. This minimizes the impact of target strand reannealing. Another important advantage is the fact that crosslinkable probes can be covalently fixed to their targets at or above the melting temperature of the probe-target complex. This minimizes secondary structure interactions and maximizes rates. Provided certain criteria are met, hybridization and crosslinking can be carried out concurrently within a matter of minutes.

b. Low Target Sequence Concentration

In laboratory experiments, purified probes and targets are used. The concentrations of these probes and targets, moreover, can be adjusted according to the sensitivity required. By contrast, the goal in the application of hybridization to medical diagnostics is the detection of a target sequence from a mixture of genomic DNA. Usually the DNA fragment containing the target sequence is in relatively low abundance in genomic DNA. This presents great technical

difficulties; most conventional methods that use oligonucleotide probes lack the sensitivity necessary to detect hybridization at such low levels.

One attempt at a solution to the target sequence concentration problem is the amplification of the detection signal. Most often this entails placing one or more labels on an oligonucleotide probe. In the case of non-radioactive labels, even the highest affinity reagents have been found to be unsuitable for the detection of single copy genes in genomic DNA with oligonucleotide probes. See Wallace et al., Biochimie 67, 755 (1985). In the case of radioactive oligonucleotide probes, only extremely high specific activities are found to show satisfactory results. See Studencki and Wallace, DNA 3, 7 (1984) and Studencki et al., Human Genetics 37, 42 (1985).

An alternate approach to the problem is to directly increase the concentration of the target prior to hybridization. Mullis et al., U.S. Patent Nos. 4,683,195 and 4,683,202, describe a method for increasing the concentration of a segment of target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a molar excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence. The two primers are complementary to their respective strands of the double-stranded target sequence. The mixture is denatured and then allowed to hybridize. Following hybridization, the primers are extended with a polymerase so as to form complementary strands. The steps of denaturation, hybridization, and polymerase extension can be repeated as often as needed to obtain a relatively high concentration of a segment of the desired target sequence. The length of the segment of the desired

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target sequence is determined by the relative positions
of the primers with respect to each other, and
therefore, this length is a controllable parameter. By
virtue of the repeating aspect of the process, the
5   method is referred to by the inventors as the
"Polymerase Chain Reaction" (hereinafter PCR). Because
the desired segments of the target sequence become the
dominant sequences (in terms of concentration) in the
mixture, they are said to be "PCR-amplified".

10   c.   Partial Complementarity
Hybridization, regardless of the method used,
requires some degree of complementarity between the
sequence being assayed (the target sequence) and the
fragment of DNA used to perform the test (the probe).
15   (Of course, one can obtain binding without any
complementarity but this binding is nonspecific and to
be avoided.) For many diagnostic applications, it is
not important to determine whether the hybridization
represents complete or partial complementarity. For
20   example, where it is desired to detect simply the
presence or absence of pathogen DNA (such as from a
virus, bacterium, fungi, mycoplasma, protozoan) it is
only important that the hybridization method ensures
hybridization when the relevant sequence is present;
25   conditions can be selected where both partially
complementary probes and completely complementary probes
will hybridize. Other diagnostic applications, however,
may require that the method of hybridization distinguish
between variant target sequences. For example, it may
30   be of interest that a particular allelic variant of a
pathogen is present. These normal and variant
sequences may differ in one or more bases.
There are other applications that may require that
the hybridization method distinguish between partial and
35   complete complementarity. It may be of interest to

detect genetic polymorphisms. Human hemoglobin is
composed, in part, of four polypeptide chains. Two of
these chains are identical chains of 141 amino acids
(alpha chains) and two of these chains are identical
5   chains of 146 amino acids (beta chains). The gene
encoding the beta chain is known to exhibit
polymorphism. The normal allele encodes a beta chain
having glutamic acid at the sixth position. The mutant
allele encodes a beta chain having valine at the sixth
10   position. This difference in amino acids has a profound
(most profound when the individual is homozygous for the
mutant allele) physiological impact known clinically as
sickle cell anemia. It is well known that the genetic
basis of the amino acid change involves a single base
15   difference between the normal allele DNA sequence and
the mutant allele DNA sequence.
        Unless combined with other techniques (such as
restriction enzyme analysis), hybridization methods
that allow for the same level of hybridization in the
20   case of both partial as well as complete
complementarity are unsuited for such applications; the
probe will hybridize to both the normal and variant
target sequence.
        Methods have been devised to enable discrimination
25   between partial and complete complementarity. One
approach is to take advantage of the temperature
requirements of the specific hybridization under study.
In typical melting curve experiments, such as those
described by Wallace et al., Nucl. Acids Res. 6, 3543
30   (1979) and Nucl. Acids Res. 9, 879 (1981), an
immobilized probe-target complex is washed at increasing
temperatures under non-equilibrium conditions. It is
observed that partially complementary probe-target
complexes display a lower thermal stability as compared
35   to completely complementary probe-target complexes.

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This difference can be used, therefore, to determine whether the probe has hybridized to the partially complementary or the completely complementary target sequence.

Conventional methods that utilize the temperature dependant nature of hybridization are artful. The application of this method for the discrimination of single base mutations in human genomic targets is limited to the use of short oligonucleotide probes where the hybridization interaction with the target sequence is in the size range of 17 bases to 25 bases in length. The lower length limit is determined by the random probability of having a complement to the probe in the human genome, which is greater than 1 for a random 16 base pair interaction, but less than 1 for interactions 17 bases or longer in length. The upper limit is one of practicality. It is difficult to differentiate single base mismatches on the basis of thermal stability for interactions longer than 25 bases in length. These conventional methods are, unfortunately, also time consuming. Probe concentrations in these experiments are approximately $1-5 \times 10^{-10}$M. These concentrations are empirically derived; they minimize the use of probe and simultaneously provide sufficient discrimination to distinguish single copy genes utilizing probes of approximately 20 nucleotides in length. Hybridization times are two to ten hours at these concentrations. After hybridization, several washes of varying stringency are employed to remove excess probe, non-specifically bound probe, and probe bound to partially complementary sequences in the target genome. Careful control of these wash steps is necessary, since the signal (specifically bound probe) to noise (non-specifically bound probe) ratio of the experiment is ultimately determined by the wash procedures.

No hybridization method heretofore described has solved all three of the problems discussed above. Crosslinkable oligonucleotide probes, while minimizing the factors of secondary structure, target strand

5   reannealing, and partial complementarily, have not solved the problems of low target concentration. Furthermore, crosslinkable probes must be used in conjunction with targets that have a crosslink side. The PCR process by itself solves the problem of low

10   target concentration. However, the specific detection of PCR products by any hybridization method is subject to the same problems associated with the detection of any target molecules. The detection of single base differences between PCR targets was initially

15   accomplished through the use of a restriction enzyme analysis of the hybridization complexes formed between oligonucleotide probes and PCR targets. More recent studies have achieved discrimination without restriction enzymes, however these studies have involved the

20   immobilization of target nucleic acids to solid surfaces (dot blot hybridization). Saiki et al., Nature 324, 163 (1986). As described in this application, the PCR process does provide a means of creating a specific crosslinking site in amplified target molecules. This

25   is obviously necessary for targets that naturally lack a crosslinking site. It is also very important for targets (such as HIV-I) which naturally undergo rapid mutation in the course of their replication. Such targets have the potential being diagnosed as absent in

30   a clinical specimen if the crosslinking site is lost by mutation.

One object of the present invention is to provide a method of hybridization that solves all three above-named problems.

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Another object of the present invention is to
provide a method of hybridization to detect the
presence or absence of a pathogen or a specific genomic
sequence in a clinical sample.

5   Another object of the present invention is to
provide a method of hybridization to detect the
presence or absence of a specific allelic variant of a
pathogen or a specific allelic variant of a genomic
sequence in a clinical sample.

10   Still another object of the present invention is
to provide a method of hybridization to determine the
genetic predisposition to a genetic disease by
identifying specific allelic variants, in clinical
samples, of nucleic acid which are genetically linked

15   to the phenotypic expression of the disease.

A further object of the present invention is to
provide a method of hybridization to determine genetic
pedigree by identifying multiply-linked allelic variants
of gene markers.

20

SUMMARY OF THE INVENTION

The present invention relates to a hybridization
method for discriminating between complementarity and
partial complementarity of DNA base sequences. The

25   method is suitable for medical diagnostic purposes in
that it overcomes the problems of low target
concentration and hybridization inefficiency. Kinetic
covalent entrapment of PCR-amplified target DNA is
described through the use of crosslinkable probes.

30   Problems with renaturation of target DNA in the
hybridization reaction are minimized. Increased target
coverage is obtained by cycling the hybridization steps
thereby pumping the equilibrium between a probe and its
target sequence.

Discrimination is shown between completely complementary probe-target sequences and probe-target sequences that differ in a single mismatch. This discrimination is achieved without the above-named

5   disadvantages of conventional allele specific hybridization, providing a practical allele specific hybridization assay for diagnostic use.

DESCRIPTION OF THE DRAWINGS

10   Figure 1 shows the primary structure of probes and targets used in the 04 human hemoglobin system.

Figure 2 shows the primary structure of probes and targets used in the 08 human hemoglobin system.

Figure 3 shows diagrammatically the method by

15   which a crosslinking site (i.e. TpA) is introduced into a PCR-product from a target genome that lacks the crosslinking site.

Figure 4 shows diagrammatically the method by which a crosslinking reagent is introduced into a PCR product.

20   Figure 5 shows the discrimination of partial complementarity and complete complementarity of DNA base sequences in solution hybridization by polyacrylamide gel electrophoresis.

Figure 6 shows the discrimination of partial

25   complementarity and complete complementarity of DNA base sequences as a function of the position of the mismatch relative to the crosslinking site in solution hybridization by polyacrylamide gel electrophoresis.

Figure 7 quantitates shows the discrimination of

30   partial complementarity and complete complementarity of DNA base sequences as a function of the position of the mismatch relative to the crosslinking site.

Figure 8 shows the enhanced discrimination achieved with a suppressor/competitor oligonucleotide

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in solution hybridization by polyacrylamide gel
electrophoresis.

Figure 9 quantifies the enhanced discrimination
achieved with a suppressor/competitor oligonucleotide
5   (Figure 8).

Figure 10 shows the DNA oligonucleotide system for
PCR amplification and subsequent detection of HIV DNA by
hybridization.

Figure 11 shows the detection of HIV DNA from PCR
10   amplified DNA of HIV-infected human cells by
polyacrylamide gel electrophoresis.

DESCRIPTION OF THE INVENTION

This invention is a means for distinguishing
15   between two target nucleic acid sequences (either DNA or
RNA) that differ by one or more bases. In one medical
diagnostic application, this invention is a means for
differentiating between a heterozygotic and homozygotic
target and, in the latter case, specifying which
20   homozygote is present. Where a given genetic locus
might code for allele A or allele a, the assay allows
for the differentiation of an AA from an Aa from an aa
pair of alleles. In another medical diagnostic
application, this invention is a means for detecting the
25   presence or absence of pathogens, as well as the
presence or absence of specific allelic variants of
pathogens, in clinical samples.

In the present invention, as with conventional
hybridization techniques, discrimination of complete or
30   partial complementarity is a function of temperature.
The temperature dependant discrimination achieved via
hybridization with crosslinkable probes was expected to
be comparable with conventional hybridization. As noted
above, the presence of the crosslinking reagent on the
35   probe does not effect the specificity of the base

pairing. The stability of the probe-target complex, furthermore, is not effected until the crosslinking step which takes place after base pairing. The only expectation of unambiguously identifying an allelic variation due to a point mutation was through the destruction of a crosslinking site. Nonetheless, the invention yields results that allow discrimination where the crosslinking site is adjacent to the site of sequence variation.

PCR-amplified targets are used. As noted above, the PCR process does not overcome the problem of reannealing of target strands during the hybridization step. In addition, the efficiency of the PCR process itself (i.e. the target production step) is hampered by reannealing. Crosslinkable probes are applied in conjunction with the PCR process such that reannealing is obviated in both of these steps.

a. Synthesis of the Probe

A photochemical synthesis of oligonucleotide probes containing site-specific psoralen monoadducts has been previously described by Gamper et al., Photochem. Photobio., 40, 20 (1984). The present invention described an improved direct chemical method for the preparation of monoadducted probes using a modification of standard phosphoramidite chemistry.

The requisite furan side cis-syn 8-MOP:thymidine monoadduct was prepared and converted to its 5'-dimethoxytrityl-3'- β cyanoethoxydiisopropyl-aminophosphoramidite derivative basically as described by Yabusaki et al., U.S. Patent 4,599,303. The yield of 5'-DMT 8-MOP:thymidine monoadduct phosphoramidite, based on starting 8-MOP:thymidine monoadduct, was approximately 50%.

DNA synthesis was conducted in a step-wise automated method on a prototype Model 510 DNA

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synthesizer (Perkin-Elmer Cetus Instruments). The solid
support used was a long-chain alkylamine controlled pore
glass (CPG) functionalized with the 3'-terminal
nucleoside derivative (American Bionetics, Hayward, CA).
5   Because of the liability of the 8-MOP:thymidine moiety
to strong aqueous base, it was necessary to make
modifications to the standard synthesis protocol. The
exocyclic amino groups of deoxyadenosine, deoxycytidine
and deoxyguanosine were protected by 9-fluorenyl-
10   methoxycarbonyl (FMOC) groups as described by Webb et
al., Nuc. Acids Res. 14, 7661 (1986), and Heikkila et
al., Acta Chem. Scand. B37, 263 (1983). The FMOC
protected nucleosides were 5'-dimethoxytritylated and
converted to the corresponding 3'-0-β-cyanoethyl-N,N-
15   diisopropyl (CED) phosphoramidites by standard
procedures as described by Beaucage et al., Tetrahedron
Letters 22, 1859 (1981). Thymidine CED phosphoramidite
was obtained from American Bionetics. The amidites were
used at an approximately thirty-fold molar excess
20   (except the monoadduct phosphoramidite, which was used
at approximately fifty-fold molar excess) and activated
prior to coupling by "Activator Gold" (Beckman
Instruments, Palo Alto, CA).
        The synthetic cycle consisted of detritylation (3%
25   v/v dichloracetic acid in dichloromethane), anhydrous
acetonitrile wash, coupling (in the dark in the case of
the psoralen containing amidite), anhydrous acetonitrile
wash, capping [1:1 mixture of A) 20% v/v 2,6-lutidine,
20% v/v distilled N-methylimidazole in dry THF, and B)
30   10% v/v acetic anhydride in THF], oxidation (0.5% w/v
iodine, 1% v/v 2,6-lutidine, 10% v/v water in THF), and
acetonitrile wash.
        Following the completion of the synthesis, the CPG
was detritylated and washed and the support from each
35   synthesis (ca. 20 mg) was dried in the dark and

suspended in 1 ml of 1M 1,8-diazabicyclo[5.4.0]undec-7-
ene (DBU) in acetonitrile for 15 minutes. This removed
the FMOC and cyanoethyl protecting groups. The CPG
washed with acetonitrile (3x1 ml) and suspended in
5   concentrated aqueous ammonia for thirty minutes. This
removed the deprotected oligomer from the CPG. The
ammoniacal solutions were passed over NAP-10 columns
(Pharmacia, Uppsalla, Sweden) which had been pre-
equilibrated with water. The eluants from the NAAP-10
10   columns were taken to dryness in the dark on the Speed-
Vac.

        Examples of two sequences prepared by the chemical
synthesis procedure are HRI-69 and HRI-70, which have
the following sequences:
15   5'-*TAG TAA GAA TGT   HRI-69
                5'- AAA *TAG TAA GAA TGT   HRI-70
                3'- TTT AGC ATT CTT ACA   HRI-68
        As shown, HRI-70 is a three base extension of
HRI-69. These sequences were selected to demonstrate
20   the ability of an oligonucleotide containing a
monoadduct at the 5' end to form crosslink and to show
the monoadduct moiety was stable to additional synthetic
cycles (i.e., DMT deprotection and oxidation steps).
Following synthesis, HRI-69 and HRI-70 were
25   characterized as follows. 5' end labelled (32P) HRI-68
(which is complementary to both HRI-69 and HRI-70) at
10-8 M was mixed with different concentrations of either
monoadducted oligonucleotide (10-8 to 10-5 M) under
hybridization conditions then irradiated at 320-400 nm
30   for 5 minutes. The samples were analyzed on a
denaturing polyacrylamide gel followed by
autoradiography. The autoradiogram showed the formation
of crosslinks between HRI-69:HRI-68 and HRI-70: HRI-68.
This result confirmed that the monoadduct
35   phosphoramidite had been incorporated during the

synthesis and some fraction of the monoadduct remained structurally intact during the subsequent synthetic steps. Second, each monoadducted oligonucleotide was 3' end labelled with cordycepin and terminal transferase then analyzed on a denaturing polyacrylamide gel. The autoradiogram showed two predominant bands for both HRI-69 and HRI-70 which corresponded to truncated 11-mer plus 12-mer monoadduct (HRI-69) and truncated 11-mer plus 15-mer monoadduct (HRI-70). The mobility of the bands with respect to the gel standards was as expected. To confirm the identity of the purported monoadduct bands, each was excised and eluted from the gel, mixed with 32P labelled HRI-68, then irradiated as described above. Each monoadduct band produced crosslink with HRI-68 as expected.

The chemical synthesis method offers several advantages over the photochemical procedure reported by Gamper et al. First, it provides milligram instead of microgram amounts of product. Second, it does not depend on the fortuitous presence of a reactive 5' TpA site in the sequence of interest. Third, it provides a method to prepare long oligonucleotides with site specific monoadducts without a ligation step.

b.   Production of Targets

The nucleic acid or acids used in the preparation of targets can be from any source, whether synthesized, cloned or naturally occurring, as long as they contain some part of the sequence of interest. In practice in a diagnostic assay, all targets are PCR amplified. In many of the specific examples given of this invention, PCR-amplified DNA fragments of naturally occurring human globin gene are used, as well as synthetic oligonucleotide targets containing sequences occurring in the human globin gene.

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Crosslinkable probes can be used to block
replication of a portion of one strand during the final
stages of PCR. Such a procedure during the production
of target step avoids the problems associated with
5   strand reannealing during the hybridization. Because
the concentration of the blocked strand is greatly
exceeded by the concentration of the strand that is
allowed to replicate, a large portion of the targets are
unable to reanneal.
10   In some applications of present invention it is
desirable to incorporate a crosslinking site in the PCR
amplified target sequence. This can be performed with
the PCR process when at least one of the primers carries
a crosslinking site as a mutation with respect to the
15   natural target sequence. For example, in the 04-MA
system (Figure 1), primer RS-118 brings in a
crosslinking site into the PCR product which is not
present in the genomic target DNA. In the 08-MA system
(Figure 2), the crosslinking site is created with primer
20   HRI-12. In both Figures 1 and 2, base pairing schemes
are indicated by the position of the oligonucleotides
(primer, probes and oligonucleotide targets) above each
other. The psoralen monoadduct is indicated by the
stick figure adjacent to the T (T). The difference
25   between the 08 system and the 04 system is the position
of the mismatch relative to the crosslinking site. In
the 04 system, the mismatch is 7 bases on the 3' side of
the psoralen monoadduct. In the 08 system, the mismatch
is 5 bases on the 5' side of the psoralen monoadduct.
30

<u>EXAMPLE 1</u>

PCR amplification is used to incorporate a psoralen
crosslinking site (i.e. 5'-TpA-3') into a segment of
amplified nucleic acid (Figure 3). One of the
35   synthetically prepared oligonucleotide primers used in

the PCR reaction is designed so that it carries a
crosslinkable dinucleotide (i.e. a 5'-TpA-3' sequence)
at a specific site. One or both of these bases are
incorporated at the time of synthesis. They replace one
5   or two natural bases in the normal primer sequence, and
thus represent either a single or double mismatch when
the primer is hybridized to its target sequence. The
sequence of this primer and the crosslinking site can be
chosen such that the crosslinking site can be
10   incorporated at a desired distance away from a potential
mutation site in a target sequence (i.e., adjacent to
the sickle cell mutation site in the globin gene). This
modified primer, along with the second normal primer,
are hybridized to a target sequence. The sequence of
15   the second primer determines the length of the amplified
PCR products. Enzymatic extension and amplification are
then carried out as usual. Upon completion of the
amplification protocol, a crosslinkable site has been
incorporated into the amplified segment of the target
20   sequence at a specifically chosen site, and the PCR
product is a specifically chosen length. This amplified
segment of nucleic acid is then probed with an
oligonucleotide containing a psoralen monoadduct at a
position complementary to the synthetically
25   incorporated crosslinkable site in the target sequence.

        In other applications of the present invention nit
is desirable to incorporate a crosslinking reagent in
the target DNA. This can also be done with the PCR
process. A crosslinking reagent, such as a triphosphate
30   of a psoralenthymine monoadduct may be incorporated
enzymatically during the extension step of the PCR
process. Alternatively, a site specific crosslinking
reagent can be incorporated. One or both of the
oligonucleotide primers can contain a crosslinking
35   reagent. Extension of these primers by PCR will

incorporate the crosslinking reagent into the longer target molecules (figure 4A). The crosslinking reagent need not necessarily be in a region of the primer required for hybridization (figure 4B). A variant of the above procedure is the use of a nested primer which is contained within the target sequence defined by the 3' ends of the original primers (figure 4C). Again, the nested primer can contain a crosslinking reagent within the segment of the nested primer that is involved in the hybridization reaction with the PCR amplified segment of the target molecule, or the crosslinking reagent can be positioned in a region of the nested primer that is not involved in the hybridization reaction with the PCR amplified segment of the target molecule. This nested primer can be extended during later cycles of the PCR process and in the presence of modified bases, such as biotin-UTP. These target molecules can then be covalently linked to conventional complementary probe molecules and subsequently detected by the presence of the modified base.

c. Hybridization Formats

Monoadducted oligonucleotide probes can be covalently fixed to PCR amplified targets, which will eliminate subsequent probe displacement which occurs when PCR strands reanneal in solution. Solving this problem is important for the development of a solution hybridization assay with double stranded target systems, and especially important for mixed phase assay systems in which the hybridization reaction is driven by high concentrations of PCR amplified targets.

EXAMPLE 2

Unexpected results were obtained from solution hybridization experiments utilizing a crosslinkable probe with two radiolabelled oligonucleotide targets: 1) a completely complementary target (normal target),

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and 2) a target with a DNA sequence that differed only
in a single base (mutant target). The sequences of the
two 31-mer targets are subsets of the 135 base pair PCR
fragments, which were generated from genomic DNA that
5   was extracted from Mut-4 and SC-1 cells, and which
represent the normal and sickle cell mutation sequences
respectively. The probe used was a monoadducted 16-mer
of the 08 system. The monoadducted probe was at a
concentration that allowed hybridization to reach
10   equilibrium in a few seconds. The hybridization buffer
consisted of 0.01M Tris (pH=7.0), 1mM EDTA, 30%
formamide, and 3.0M tetramethylammonium chloride. A
chaotropic salt was used to minimize sequence specific
effects on the hybridization interactions. The targets
15   were denatured, placed at the hybridization temperature,
and irradiated at this temperature. Many hybridization
temperatures were used between 4 and 40°C (the products
at each temperature are shown in each gel lane). The
products of the hybridization were isolated by
20   precipitation and centrifugation and then
electrophoresised on a denaturing polyacrylamide gel
which was analyzed by autoradiography. The results are
shown in Figure 5. The free (uncomplexed) ^{32}P-labelled,
31-mer targets run to the bottom of the gel. The
25   probe:mutant target complex (XL_m) is retarded in the
gel, running above the probe:normal target complex
(XL_n). It is clear that the melting temperature for the
mutant target was 12°C lower than the melting
temperature of the normal target.
30   It is not yet known whether this large melting
temperature difference represents an enhancement brought
about by the presence of the crosslinking reagent. It
may be due to the fact that hybridization (and
crosslinkage) under these conditions occurs at
35   equilibrium in contrast to conventional hybridization,

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where discrimination occurs under non-equilibrium
conditions. At equilibrium and above the melting
temperature of the probe:mutant target complex, there
is a strong preference for the probe to bind
5   preferentially to the normal target. This preference
may depend upon the position of the crosslinking site in
the probe:target complex. Indeed, it may be possible to
enhance discrimination between normal and mutant target
sequences by positioning the crosslinking site at a
10   precise position relative to the mismatch site of the
probe:target complex. This can be accomplished through
the use of specifically chosen PCR primers.
        Figure 5 also demonstrates that covalent
probe:target complexes of identical molecular weight can
15   be resolved by standard polyacrylamide gel
electrophoresis (PAGE) when one of the complexes
contains at least a single base mismatch between the
target and the probe. This resolution requires that
both the normal and mutant target oligonucleotides be of
20   identical length, and that the crosslinking site be
precisely positioned relative to the mismatch site in
the probe target complex. Again this is accomplished
through the use of specifically chosen PCR primers.

25   EXAMPLE 3
        Figures 6 and 7 illustrate the best mode of
carrying out the invention. Discrimination is measured
as a function of the position of the mismatch relative
to the crosslinking site. Both the 08 system and the 04
30   system were used. Discrimination was measured at either
4°C or 27°C -- temperatures at which both hybridization
and crosslinkage were conducted. Unirradiated samples
were run as controls. The conditions were in other
respects (buffer, isolation steps, gel electrophoresis,
35   etc.) similar to those in Example 2. Figure 6a and 6b

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show the autoradiogram of the gel products of the
hybridization reaction conducted at either 4°C or 27°C,
respectively. It is to be noted that in both Figure 6a
and 6b, the migration of 04 probe crosslinked to HRI-10
5   targets is the same as the migration of 04 probe
crosslinked to HRI-11 targets. In other words, the
probe:mutant(single base mismatch) target complex
cannot be distinguished from the probe:normal target
complex using the 04 probe. On the other hand, the
10   probe:mutant target complex is distinguished from the
probe:normal target complex (08-MA XL HRI-16) using the
08 probe.

The ability to resolve these products is the basis
of discriminating between having a point mutation or
15   containing normal DNA in the sequence region of
interest. When either of these bands are present alone
in a gel analysis of the probe:target crosslinked
products, the individual in question would be considered
homozygous for either the normal or the mutation,
20   depending upon which band was present. When both bands
are present, the individual would be considered a
heterozygote.

For the purposes here, discrimination is defined
as the ratio of a normal probe bound to a normal target
25   relative to the normal probe bound to a mutant target
under identical equilibrium conditions. Discrimination
was measured with the 08-MA system. The bands from
Figures 6a and 6b were simply excised and counted. The
results are summarized in Figure 7. In addition to the
30   requirements of 1) a psoralen monoadducted probe, and 2)
a target molecule of a short and precise length, Figure
7 shows that the best discrimination is achieved when
the mutation in question is orientated in a specific
site relative to the crosslinking site. For most types
35   of gel analysis, discrimination (as strictly defined

above) should be minimized, i.e. the amount of normal
probe bound to a normal target and normal probe bound to
a mutant target should be approximately equal. This
allows for the simultaneous identification of each
5   product with a single probe if both are present. For
other types of analysis, it may be desirable to maximize
discrimination, i.e. establish conditions where either
the normal or the mutant are resolved.

<center>EXAMPLE 4</center>

10   Another method of practicing the invention
involves attaching the probe to a solid support.
Specifically, polystyrene beads with surface carboxylic
acid groups were coupled to 4'-hydroxymethyl-4,5', 8-
trimethylpsoralen monoadducted probe (a 16-mer, HRI-23,
15   with an aminopolyether tail at the 5' end) using a water
soluble carbodiimide. The method of attachment is
described generally by Kremsky, J. et al., Nucleic Acids
Research 15, 2891 (1987).

        Target nucleic acids from MUT-4 cells (normal
20   sequence) and SC-1 cells (sickle cell sequence) were
prepared by PCR amplification using primer molecules
RS-118 and KM-38. The two PCR product sequences differ
by a single base. Following PCR, each fragment was
isolated on an 8% denaturing polyacrylamide gel and
25   labelled by kinasing.

        Experimental points utilized 50 ul of a 3% bead
solution which contained 38 fmol/ul of monoadducted
probe. The final probe concentration was 4×10^{-8} M.
Either MUT-4 or SC-1 PCR product was added to each
30   sample tube at a concentration of approximately
2×10^{-10} M.

        Each experimental sample (two per set) was
irradiated five cycles. One cycle consists of a)
denaturation carried out at 85°C for 5 minutes, b)
35   hybridization carried out at 30°C temperature for 3

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minutes and c) irradiation carried out at 30°C for 5
minutes. The control samples (one per set) were
identical to the experimental samples but were not
irradiated. Following irradiation, the beads were
5   washed (five times at 50°C in 50mM Na_2HPO_4/0.05% SDS).
At this point either 1) the captured targets were
recovered by photoreversal of the crosslinking
reaction, or 2) the beads were counted in scintillation
fluid. In the latter case, bead recovery was determined
10   by tritium counts while PCR fragment capture was
determined by ^{32}P. Under these conditions,
discriminatory capture was 23:1 in favor of the normal
sequence.

EXAMPLE 5

15   The bead experiment described in Example 4 was
performed in the same manner except that the
polyacrylamide gel isolation step was omitted. The
beads containing probes were added directly to the PCR
amplified targets.

EXAMPLE 6

20   The bead experiment described in Example 4 was
performed in much the same manner except that the beads
used were magnetic beads.

EXAMPLE 7

25   Another method of practicing the invention involves
the use of RNA. DNA targets are made from RNA templates
using reverse transcriptase. Murakawa et al., DNA 7,
287 (1988). Thereafter, the DNA targets are used in the
30   amplification and hybridization steps in the same way as
Examples 2, 4, 5 or 6.

EXAMPLE 8

Teflon was substituted as the solid support in
35   EXAMPLE 4. A cleavable teflon support can be obtained

from Molecular Biosystems, Inc. The oligonucleotide is
attached to the support at its 3' end with a 25 atom
spacer in between. The steps are then carried out in
much the same way as in EXAMPLE 4.

5

EXAMPLE 9

In the experiments described in Examples 2 and 4-7,
chemical crosslinking may be used instead of
photochemical crosslinking. Webb et al. Nuc. Acids Res.
10   14; 7661 (1986).

EXAMPLE 10

In the experiments described in Examples 2 and 4-7,
a combination of chemical crosslinking and photochemical
15   crosslinking may be used.

EXAMPLE 11

In a situation where a single fragment of target
DNA contains two or more sites of allelic variation,
20   the fragment may be simultaneously probed with two
allele specific crosslinkable probes. The products of
such a hybridization may be examined by any of the
methods used above. Given two sites with two sequence
patterns at each site, the possible linkage patterns
25   which will emerge are AB, aB, Ab, or ab. The power of
such a procedure for clinical characterization and
parental testing as the number of sites increases and
the variation at individual sites increases is
considerable.

30

The examples given above demonstrate that it is
possible using the 08 system to quantitatively measure
discrimination of a single mismatch in a hybridization
assay with short crosslinkable probes. It is possible
35   to further increase this discrimination through the

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addition of a "suppressor" oligonucleotide which is
complementary to the target oligonucleotide that one
desires not to capture. This approach is based on the
empirical observation that normal probe (i.e. probe
5   having the normal sequence) preferentially binds to
normal targets and mutant probe (i.e. probe having the
mutant sequence) preferentially binds to mutant target.

        Specifically, a mutant probe lacking crosslinking
reagents and a normal, monoadducted probe are introduced
10   to the target preparation. Binding of the normal probe
to mutant targets is blocked since these mutant target
molecules have a thermodynamic preference for the mutant
probes. This type of assay can be used with a bead (or
membrane format) where the beads (or membrane) are
15   coupled to a mixture of normal probes and mutant probes,
only one of which contains a crosslinkable group. This
type of assay can also be performed in a solution format
and analyzed on gels as described below.

20   EXAMPLE 12

        Discrimination is enhanced via a suppressor
oligonucleotide. The synthetic oligonucleotides from
the 08 system were used; HRI-08 and HRI-08-MA (normal
probes), HRI-19 and HRI-19-MA (mutant probes), HRI-16
25   (a normal 31-mer target), and HRI-17 (a mutant 31-mer
target). HRI-16 and HRI-17 were ^{32}P labelled and
purified by electrophoresis before use.

        Samples were prepared in a chaotropic salt buffer
(1X TE buffer, 30% formamide, 3.0M tetramethylammonium
30   chloride) with a total volume of 10 ul. The labelled
targets (both HRI-16 and HRI-17) were used at 1×10^{-8}M.
Probe molecules (probes with and without crosslinking
reagents) were used at 3.7×10^{-8}M. After preparing the
samples, they were heat denature at 50°C for 5 minutes.
35   They were hybridized for 1 minute at the indicated

temperatures, and then irradiated at this same
temperature for 5 minutes. All the samples were ethanol
precipitated and then analyzed on a 20% polyacrylamide,
7M urea gel.

5   Figure 8 is an autoradiogram showing the
crosslinked products as a function of hybridization and
irradiation temperature. In panel A, only a
monoadducted normal probe (08-MA) was present during
the hybridization. This probe crosslinks to both the
10   normal and the mutant targets, with a preference for
the normal target. In panel C, only a monoadducted
mutant probe (19-MA) was present during the reaction.
Again this probe hybridizes to both its completely
complementary sequence and the partially complementary
15   sequence. In complete analogy with the 08-MA probe, the
mutant probe (19-MA):normal target complex is retarded
in the gel with respect to the position of the mutant
probe crosslinked to the normal target.

        In Panel B, a monoadducted normal probe (08-MA)
20   was used in conjunction (1:1 ratio) with a mutant probe
(19) lacking crosslinking reagents. Very little of the
08-MA probe was observed to crosslink with the mutant
target. The 08-MA probe, however crosslinked to the
normal target to the same extent as it did in the
25   absence of the suppressor oligonucleotide (19).

        To quantify this effect, the crosslinked bands
were excised and counted. The fraction of the targets
hybridized and crosslinked was determined at each
temperature, and from this data discrimination was
30   determined (ratio of normal probe/normal target
complexes to the normal probe/mutant target complexes).
This data is plotted in Figure 9.

        Figure 9 shows that the additional "suppressor"
oligonucleotide enhances discrimination, particularly at
35   temperatures below the melting temperature of the

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completely complementary complex (28°C). The relative
fraction of target molecules hybridized is also shown.
With conventional equilibrium conditions, discrimination
is sharply dependant upon temperature, with maximum
5   discrimination occurring slightly above the melting
temperature of the completely complementary probe
target complex. At this temperature the relative
fraction of target molecules hybridized is only about
25%. When the additional suppressor oligonucleotide is
10   present, discrimination is relatively insensitive to
hybridization temperatures between the melting
temperature of the completely complementary probe
target complex (28°C) and the melting temperature of
the partially complementary probe target complex
15   (16°C). Within this temperature region, the relative
hybridization efficiency is constant and maximum (near
100%). Discrimination is also maximum in this region.
This technique (equilibrium hybridization/crosslinking
with a mixture of crosslinkable and non-crosslinkable
20   probes), therefore, provides a relatively temperature
insensitive procedure for identifying point mutations.

As noted earlier, it may be of interest to detect
the presence or absence of pathogen DNA (such as from a
25   virus, bacterium, fungi, mycoplasma, protozoan). It may
also be of interest that a particular allelic variant of
a pathogen is present. These normal and variant
sequences may differ in one or more bases. The
invention provides a method for such discrimination. At
30   present, there is great interest in detecting Human
Immunodeficiency Virus (HIV). Most methods now in
clinical use measure HIV only indirectly; such methods
are aimed at detecting host antibodies elicited in
response to infection. Methods aimed at directly
35   detecting HIV DNA are hampered by the presence of 1) an

extremely high mutation rate associated with HIV, and
2) extremely low levels of viral DNA in clinical
samples. The present invention overcomes these
problems. Of particular importance to the former
5 problem is the ability with the present invention to
introduce crosslinking sites into the targets at
specific sites during the amplification step. This
ensures the existence of a crosslinking site in the
sequence of a PCR amplified target. Of particular
10 importance to the latter problem is the ability with the
present invention to cycle the hybridization steps
(denaturation/irradiation) thereby pumping the
equilibrium to increase the coverage and, consequently,
the signal.

15

EXAMPLE 13

DNA template was obtained from blood samples taken
from human patients known to be positive for HIV-I.
The DNA oligonucleotide system for PCR amplification and
20 subsequent detection of HIV DNA by hybridization with
SK-19 or SK-19-MA is shown in Figure 10. The arrows
indicate the polymerase extension direction for the
primers. The HMT monoadduct on SK-19-MA is shown by
( ). Blocks □ denote natural potential 5-TpA-3'
25 crosslinking sites in the DNA sequence of HIV.

10 ul of template was amplified using primer pair
SK-38/SK-39 for thirty cycles. Following amplification,
3.3 ul of monoadducted 32P labelled 41-mer probe was
added (SK-19-MA) at a final concentration of 8×10^{-9} M.
30 Following a 5 minute heat denaturation at 90-95°C,
samples were irradiated at 55°C for 5 minutes. This
denaturation/irradiation cycle was repeated two more
times. For analysis, samples were heat denatured and
then run on a denaturing (8M urea) 12% polyacrylamide
35 gel.

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As controls, the same experiment was done on an 1) amplified, unirradiated sample; 2) irradiated, unamplified sample, and 3) unirradiated, unamplified sample.

Figure 11 is an autoradiogram of the results. Lanes marked "M" are markers, showing unmodified 41-mer (SK-19) and 71-mer (the latter marker not pertinent here). Lane 1 is unirradiated 41-mer monoadducted probe (SK-19-MA) alone. Lane 2 is irradiated amplified sample plus probe. Lane 3 is unirradiated amplified sample plus probe. Lane 4 is irradiated unamplified sample plus probe. Lane 5 is unirradiated unamplified sample plus probe.

The autoradiograph shows 115-mer:41-mer cross-linked product in Lane 2 only. Thus, the generation of signal is shown to depend on both irradiation and amplification. Most importantly, the autoradiograph demonstrates the resolution on a denaturing polyacrylamide gel of free monoadducted probe (SK-19-MA) and the covalent complex between the monoadducted probe and the 115-mer generated by PCR amplification of DNA extracted from HIV-infected human cells.

CLAIMS

1.   A method for discriminating between two or
more nucleic acid base sequences in one or more nucleic
acid target molecules comprising:

  a.   providing one or more single stranded
nucleic probe molecules which are characterized as
having at least one complementary base sequence to
at least one of said nucleic acid base sequences of
said one or more nucleic acid target molecules;

  b.   providing a crosslinking reagent capable
of forming covalent bonds between said nucleic acid
target molecules and said single stranded nucleic
acid probe molecules;

  c.   providing a label to any one of said (i)
nucleic acid target molecules, (ii) single stranded
nucleic acid probe molecules or (iii) crosslinking
reagent;

  d.   denaturing said nucleic acid target
molecules and single stranded nucleic acid probe
molecules;

  e.   hybridizing said single stranded nucleic
acid probe and nucleic acid target molecules at one
or more distinct temperatures in the presence of
said crosslinking reagent;

  f.   crosslinking said crosslinking reagent to
form covalent bonds between said one or more single
stranded nucleic acid probe molecules and said one
or more nucleic acid target molecules at one or
more locations where hybridization has occurred so
as to create probe:target complexes; and

  g.   identifying said label as a measurement
of the amount of covalently crosslinked single
stranded probe and nucleic acid target molecules.

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2.   The method as recited in claim 1 where said crosslinking reagent in sub-part E is unattached in solution.

5   3.   The method as recited in claim 1 where said crosslinking reagent is attached to one or more of said probe molecules prior to the hybridization step of sub-part E.

10   4.   The method as recited in claim 1 where said crosslinking reagent is attached to one or more of said target molecules prior to the hybridization step of sub-part E.

15   5.   The method of claim 1 further comprising, prior to the measuring step of sub-part G, repeating the steps of sub-parts D, E, and F one or more times.

6.   The method as recited in claim 1 wherein said
20   crosslinking reagent is psoralen.

7.   The method of claim 6 wherein said psoralen is a member selected from the group consisting of 8-methoxypsoralen, 5-methoxypsoralen, 4,5',8-trimethyl-
25   psoralen, 4'-hydroxymethyl-4,5',8-trimethylpsoralen, and 4'-aminomethyl-4,5',8-trimethylpsoralen.

8.   The method as recited in claim 1 wherein said label is chosen from among radioactive labels, immunogenic labels, chromogenic labels, and fluorogenic labels.

9.   The method as recited in claim 1 wherein said covalent bonds are formed photochemically.

10.   The method of claim 1 further comprising, prior to the step of sub-part D, attaching said one or more probe molecules to a solid support.

11.   The method of claim 1 further comprising, prior to the step of sub-part D, attaching said one or more target molecules to a solid support.

12.   The method as recited in claim 10 or 11 wherein said solid support consists of functionalized polystyrene having surface groups appropriate for activation and coupling.

13.   The method as recited in claim 10 or 11 wherein said solid support is tetrafluoroethylene.

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14. The method of claim 1 further comprising, prior to the step of sub-part B, attaching said one or more probe molecules to a membrane support.

5   15. The method of claim 1 further comprising, prior to the step of sub-part C, attaching said one or more target molecules to a membrane support.

16. The method as recited in claim 1 wherein said
10   denaturing is achieved by exposure to high temperature.

17. The method of claim 1 further comprising, prior to the measuring step of sub-part G, a means for separating uncrosslinked probe molecules and target
15   molecules from crosslinked probe molecules and target molecules.

18. The method of claim 1 further comprising, prior to the measuring step of sub-part G, a means for
20   separating crosslinked probe:target complexes that differ by a single base substitution in the target sequence.

19. The method as recited in claim 17 or 18
25   wherein said separation means consists of polyacrylamide gel electrophoresis.

20.   The method of claim 1 further comprising the
step of photoreversal of the crosslinked probes and
targets.

5

21.   The method of claim 20 further comprising
recovery of the photoreversed products.

22.   The method as recited by claim 21 wherein said
10   recovery consists of polyacrylamide gel electrophoresis
and elution of the photoreversed products from the gel.

23.   The method as recited in claim 1 where said
target molecules are obtained by a process comprising:
15   a.   treating a sample containing a nucleic
acid or a mixture of nucleic acids with one
oligonucleotide primer for each strand of each different
specific sequence, under hybridizing conditions such
that for each strand of each different sequence to which
20   an oligonucleotide primer is hybridized an extension
product of each primer is synthesized which is
complementary to each nucleic acid strand, wherein said
primer or primers are selected so as to be sufficiently
complementary to each strand of each specific sequence
25   to hybridize therewith such that the extension product
synthesized from one primer, when it is separated from

its complement, can serve as a template for synthesis of
the extension product of the other primer;

        b.   treating the sample under denaturing
conditions to separate the primer extension product from

5. their templates if the sequence or sequences to be
detected are present;

        c.   treating the sample with oligonucleotide
primers such that a primer extension product is
synthesized using each of the single strands produced in

10 step (b) as a template, resulting in amplification of
the specific nucleic acid sequence or sequences if
present; and ———

        d.   repeating the steps (b) and (c) at least
once.

15

    24.   The method of claim 23 wherein at least one of
said primers contains a crosslinking site so that at
least one crosslinking site is introduced into the
extension product and at least one crosslinking site is

20 introduced into the resulting amplified specific nucleic
acid sequence or sequences.

    25.   The method of claim 23 wherein at least one of
said primers contains a crosslinking reagent.

25

26. The method of claim 23 further comprising, prior to the last repetition of sub-part d;

    a.   treating the sample with a crosslinkable probe that is complementary to one of said strands under hybridizing conditions; and

    b.   crosslinking said crosslinkable probe to said complementary strands if present so as to prevent replication and amplification of said complementary strand.

PCR PRODUCT OF NORMAL (β^N) GLOBIN = 135 bp; MW = 88,100

PCR PRIMERS

RIGHT PRIMER (C)   3'-GTTCTGTCCAAATTCCTCTGGT-5' ⇒   KM-38

5'-AC ACC ATG GTG CAC CTT ACT-3'   LEFT PRIMER (W)   RS-118

GENOMIC DNA SEQUENCE OF β^N

5'-AC ACC ATG GTG CAC CTG ACT CCT GAG GAG AAG TCT GCC GTT ACT GCC-3'   WATSON (W)

3'-TG TGG TAC CAC GTG GAC TGA GGA CTC CTC TTC AGA CGG CAA TGA CGG-5'   CRICK (C)

MONOADDUCTED PROBE

5'- C CTT ACT CCT GAG GAG -3'   HRI-04

SYNTHETIC OLIGONUCLEO- TIDE TARGETS

3'- G GAA TGA GGA CTC CTC TTC AGA CGG CAA TGA -5'   HRI-10

3'- G GAA TGA GGA CAC CTC TTC AGA CGG CAA TGA -5'   HRI-11

3'- G GAA TGA GGA TTC CTC TTC AGA CGG CAA TGA -5'   HRI-20

HRI 04-MA SYSTEM

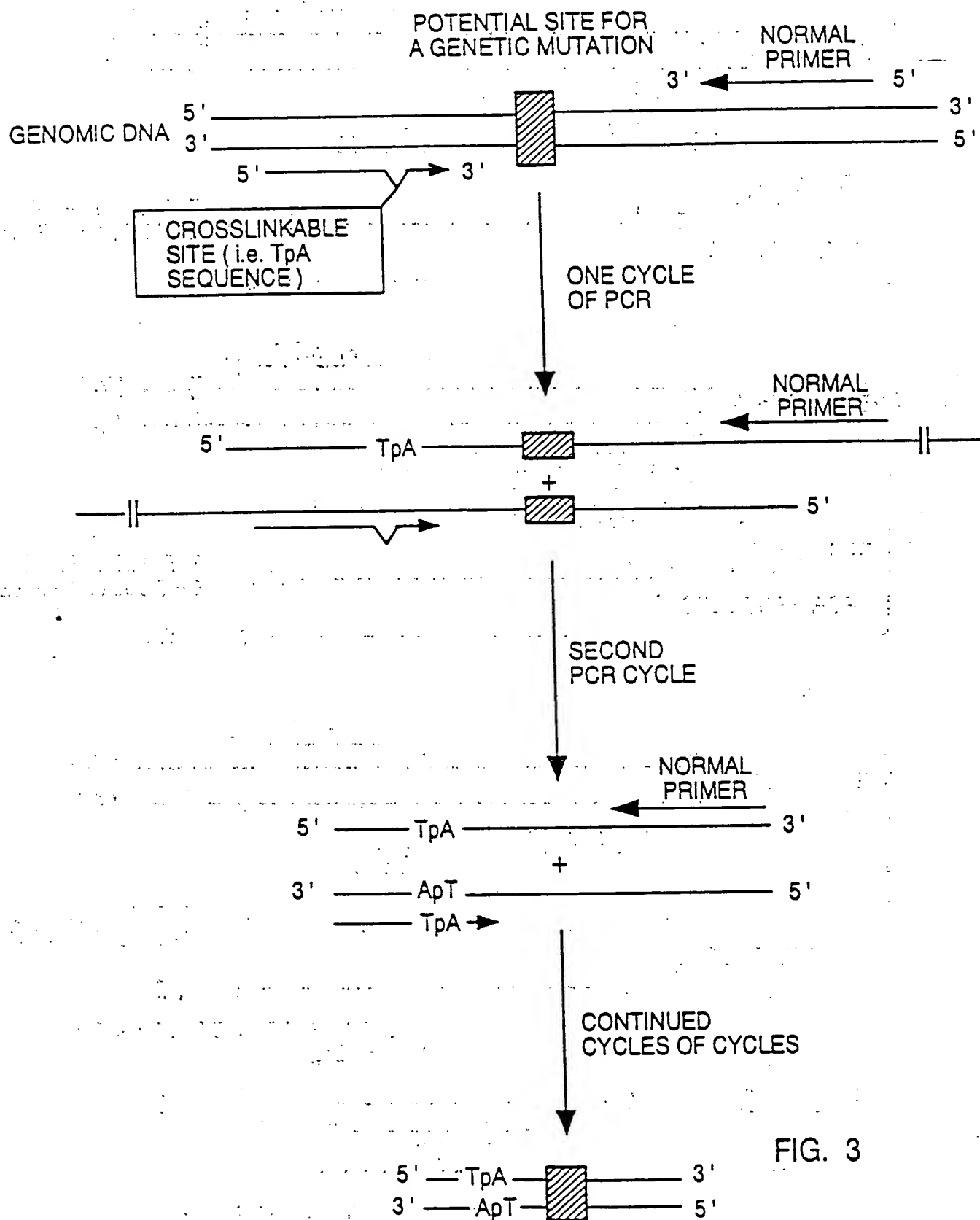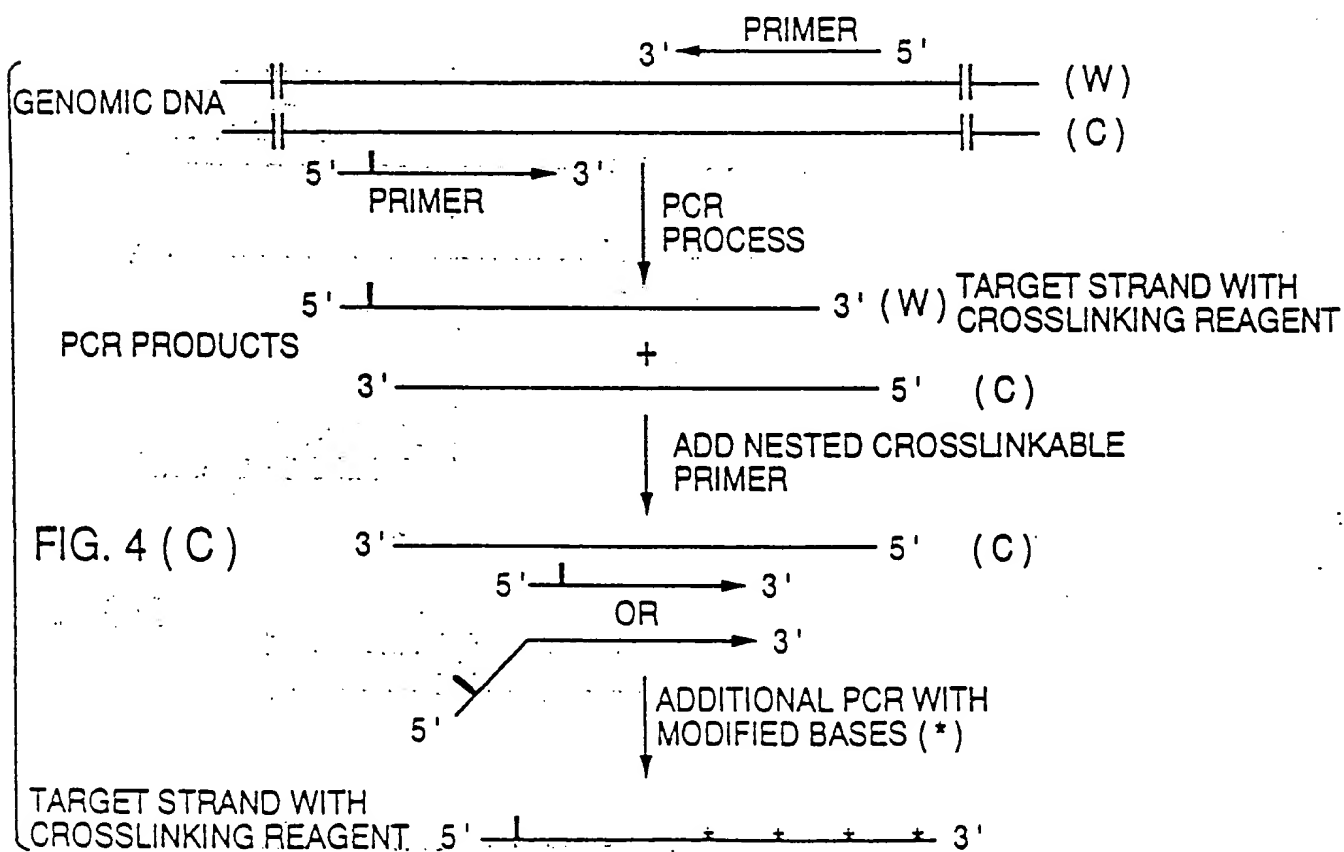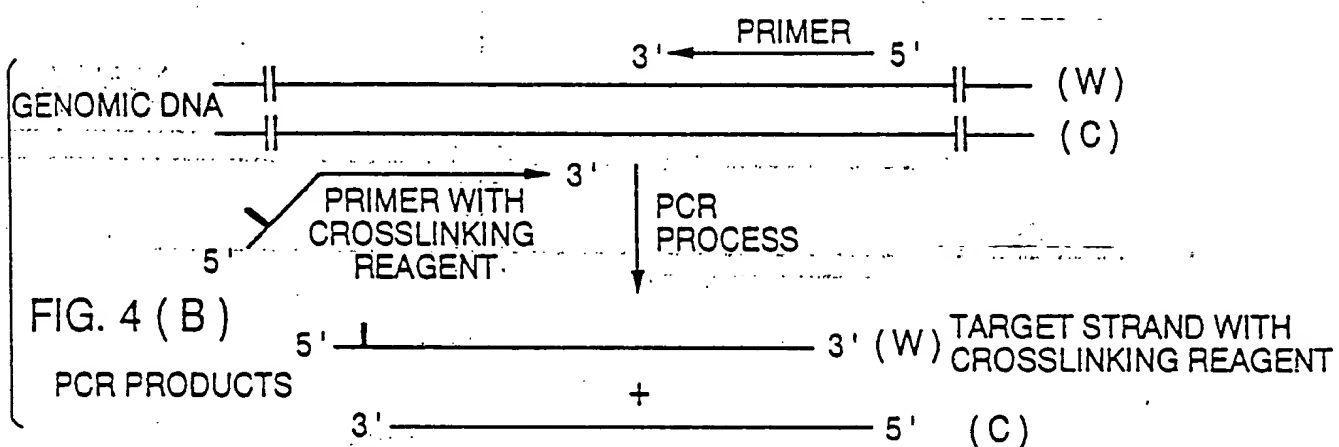FIG. 1

PCR PRODUCT OF NORMAL (β^N) GLOBIN = 174 bp; MW = 114,840

PCR PRIMERS

KM-29   ⇐ 5'-GGTTGGCCAATCTACTCCCAGG-3'

LEFT PRIMER (W)

IIRI-12   3'-TC ATC AGA CGG CAA TGA CGG-5'

RIGHT PRIMER (C)

GENOMIC DNA SEQUENCE OF β^N

WATSON (W)   5'-AC ACC ATG GTG CAC CTG ACT CCT GAG GAG AAG TCT GCC GTT ACT GCC-3'

CRICK (C)   3'-TG TGG TAC CAC GTG GAC TGA GGA CTC CTC TTC AGA CGG CAA TGA CGG-5'

MONO-ADDUCTED PROBES

N-PROBE (W)   5'- CCT GAG GAG T/AG TCT G -3'

S-PROBE (W)   5'- CCT GTG GAG T/AG TCT G -3'

SYNTHETIC OLIGONUCLEO-TIDE TARGETS

IIRI-08 MA
IIRI-19 MA

IIRI-16   3'- G GAC TGA GGA CTC CTC ATC AGA CGG CAA TGA -5'
IIRI-17   3'- G GAC TGA GGA CAC CTC ATC AGA CGG CAA TGA -5'
IIRI-21   3'- G GAA TGA GGA CTT CTC ATC AGA CGG CAA TGA -5'
IIRI-25   3'- G GAC TGA GGA CTC CTT ATC AGA CGG CAA TGA -5'
IIRI-26   3'- G GAC TGA GGA CTC CTC CAC ATC AGA CGG CAA TGA -5'
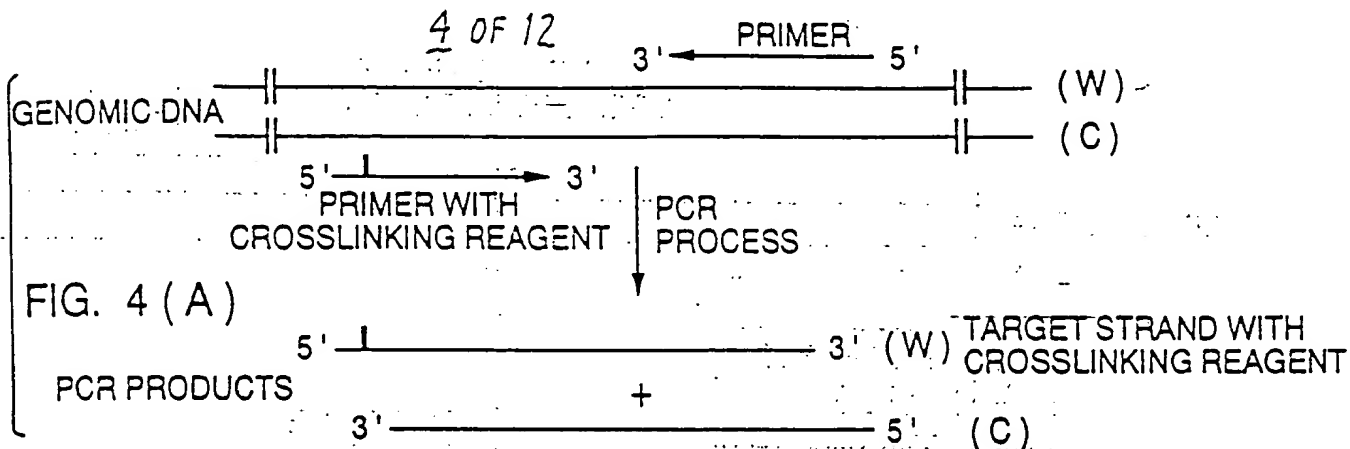
FIG. 2   IIRI 08-MA SYSTEM

POTENTIAL SITE FOR
A GENETIC MUTATION

NORMAL PRIMER

GENOMIC DNA

CROSSLINKABLE SITE (i.e. TpA SEQUENCE)

ONE CYCLE OF PCR

NORMAL PRIMER

SECOND PCR CYCLE

NORMAL PRIMER

CONTINUED CYCLES OF CYCLES

FIG. 3

FIG. 4 (A)

PRIMER

GENOMIC DNA (W) (C)

PRIMER WITH CROSSLINKING REAGENT

PCR PROCESS

PCR PRODUCTS

5' — 3' (W) TARGET STRAND WITH CROSSLINKING REAGENT

+

3' — 5' (C)

FIG. 4 (B)

PRIMER

GENOMIC DNA (W) (C)

PRIMER WITH CROSSLINKING REAGENT

PCR PROCESS

PCR PRODUCTS

5' — 3' (W) TARGET STRAND WITH CROSSLINKING REAGENT

+

3' — 5' (C)

FIG. 4 (C)

PRIMER

GENOMIC DNA (W) (C)

PRIMER

PCR PROCESS

PCR PRODUCTS

5' — 3' (W) TARGET STRAND WITH CROSSLINKING REAGENT

+

3' — 5' (C)

ADD NESTED CROSSLINKABLE PRIMER

3' — 5' (C)

5' — 3'

OR

3'

5'

ADDITIONAL PCR WITH MODIFIED BASES (*)

TARGET STRAND WITH CROSSLINKING REAGENT   5' — * * * * 3'

FIG. 5

FIG. 6(A)

FIG. 6 (B)

28°C

XL_M

XL_N

XL_{N} XL_M

31mer
Targets

TYPE AND LOCATION OF SINGLE MISMATCH

	TARGET OLIGO	PROBE OLIGO	HYBRID & IRRAD. TEMP.	FRACTION HYBRIDIZD	DISCRIM-INATION
CONTROL	HRI 10	04-MA	4°C	0.252	----
A/A	HRI 11	04-MA	4°C	0.039	6.5
CONTROL	HRI 10	04-MA	27°C	0.336	----
A/A	HRI 11	04-MA	27°C	0.026	12.9
CONTROL	HRI 16	08-MA	4°C	0.677	----
A/T	HRI 17	08-MA	4°C	0.312	2.2
A/G	HRI 21	08-MA	4°C	0.243	2.8
A/A	HRI 26	08-MA	4°C	0.454	1.5
T/G	HRI 25	08-MA	4°C	0.661	1.0
CONTROL	HRI 16	08-MA	27°C	0.620	----
A/T	HRI 17	08-MA	27°C	0.083	7.5
A/G	HRI 21	08-MA	27°C	0.087	7.1
A/A	HRI 26	08-MA	27°C	0.318	1.9
T/G	HRI 25	08-MA	27°C	0.468	11.3

04 PROBE: 5'-CCTTACTCCTGAGGAG-3'

GENOMIC TARGET SEQUENCE: 3'-GGACTGAGGACTCCTCTTCAGACGGCAATGA-5'

08 PROBE: 5'-CCTGAGGAGTAGTCTG-3'

FIG. 7

FIG. 8

FIG. 9

FIG. 10

SK38: 5' - AT AAT CCA CCT ATC CCA GTA GGA GAA AT - 3'

SK39: 5' - TT TGG TCC TTG TCT TAT GTC CAG AAT GA - 3'
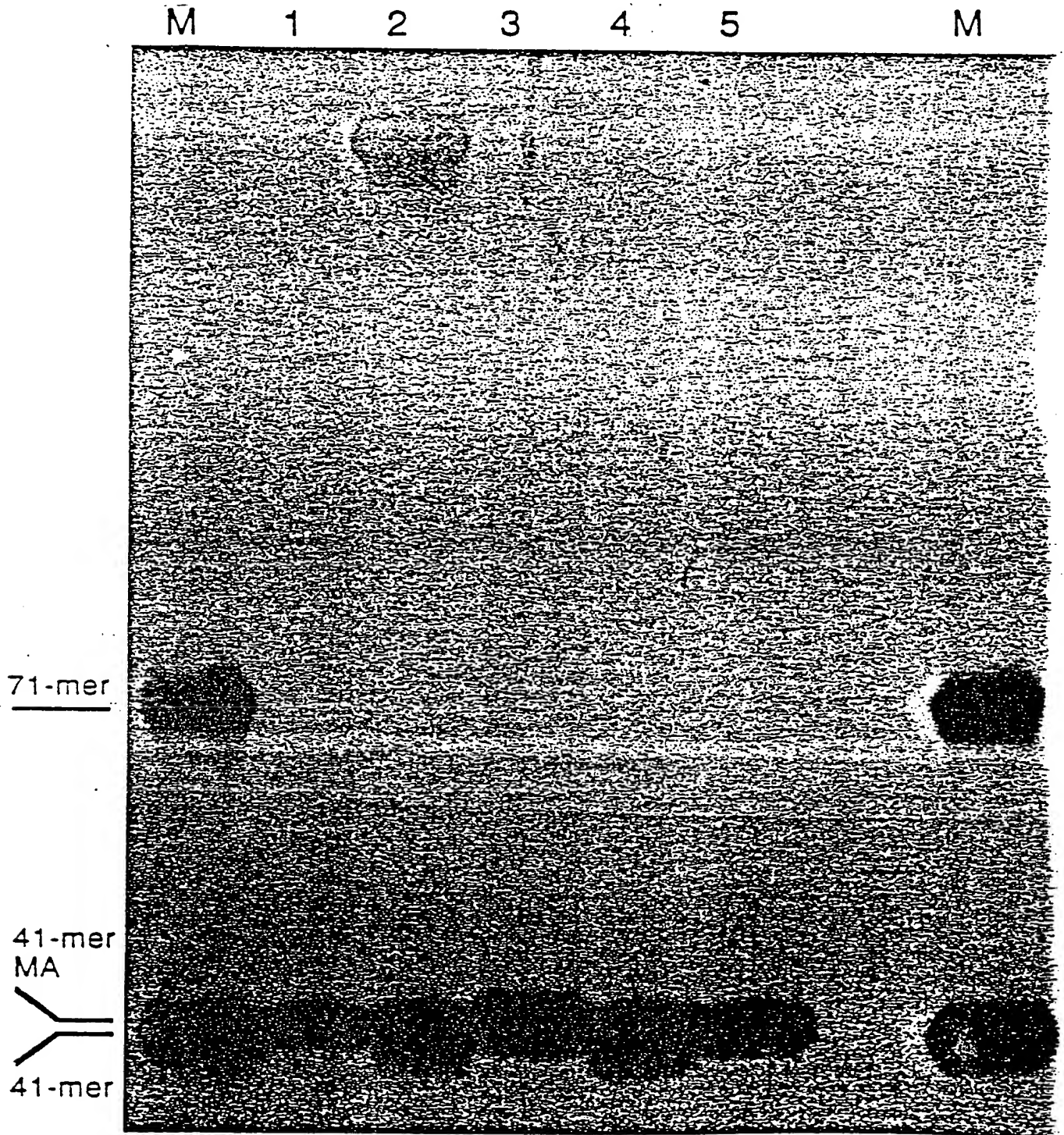
PCR TARGET = 115mer

FIG. 11

M   1   2   3   4   5   M
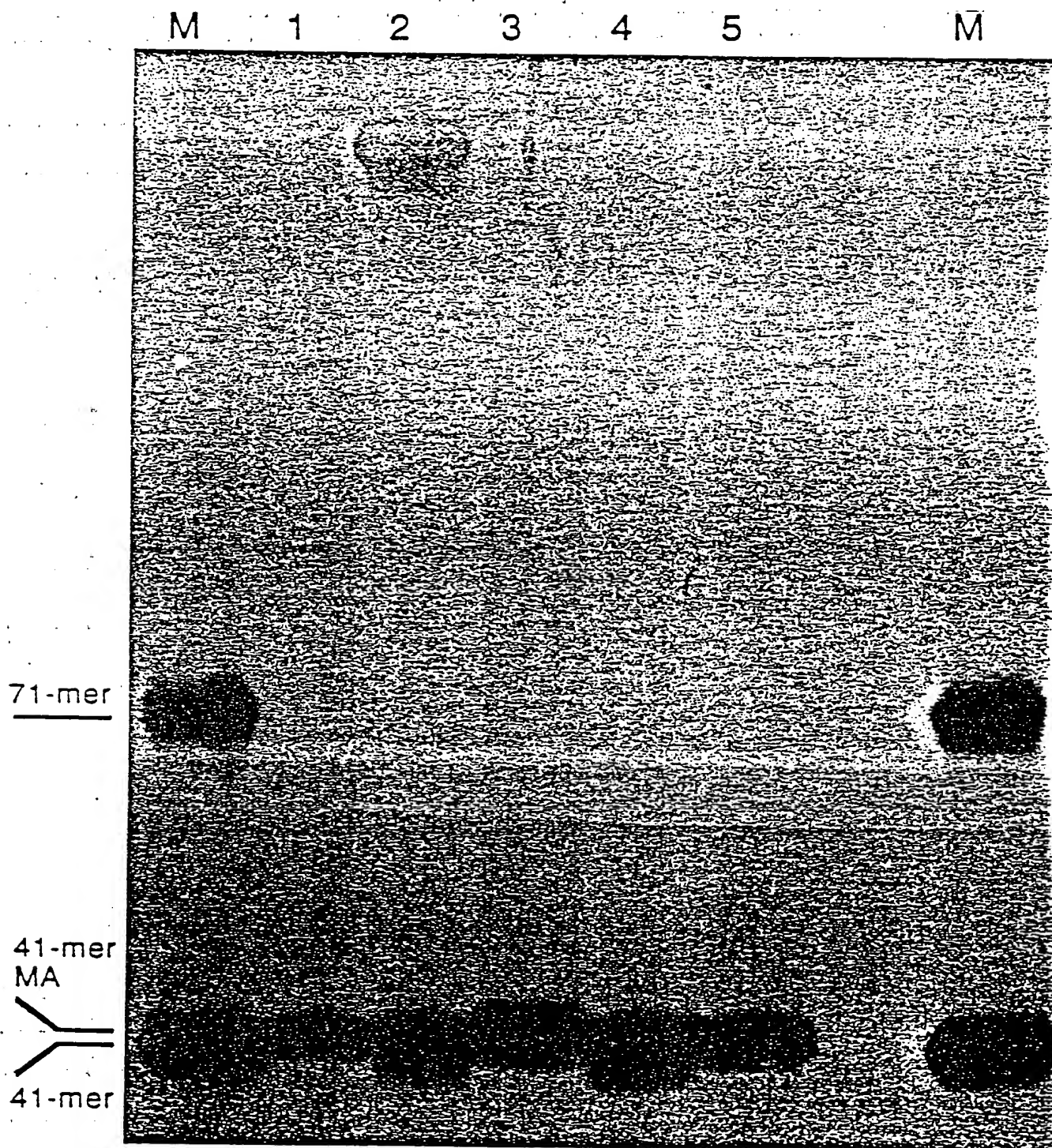
71-mer

41-mer
MA

41-mer

FIG. 11

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No **PCT/US89/03189**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12 Q 1/68
U.S. Cl.: 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/6,91,803 548/303 436/501 935/19,78 536/27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US, A, 4,599,303 (YABUSAKI, ET AL.) 08 July 1986 (08.07.86), See Abstract and Column 2, line 24 through column 5, line 25.	1-22
Y	US, A, 4,683,195 (MULLIS, ET AL.) 28 July 1987 (28.07.87), See Abstract.	23-26
Y	US, A, 4,683,202 (MULLIS) 28 July 1987 (28.07.87), See Abstract.	23-26
Y	EP, A, 0,130,523 (DATTAGUPTA, ET AL.) 09 January 1985 (09.01.85), See Claims 1-10.	1-15
Y	Photochemistry and Photobiology, Volume 40, published in 1984 (London, England), H. Gamper et al., "Efficient Formation of a Crosslinkable HMT Monoadduct at the Kpn I Recognition site," pages 29-34, see especially the Abstract and Figure 3.	1-9, 19-21
Y	Science, Volume 197, published in 1977 (Washington D.C.) B. Johnston, et al., "Psoralen-DNA Photoreaction: Controlled Production of Mono- and Diadducts with	17

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 November 1989 (14.11.89)	14 DEC 1989

International Searching Authority	Signature of Authorized Officer
ISA/US	Ardin Marschel

	Nanosecond Ultraviolet Laser Pulses," pages 9●●908, see especially Figu●●1 on page 906.	
X	Nucleic Acids Research, Volume 14, published in 1986 (Oxford, England) H. Gamper, et al., "Reverse Southern Hybridization," pages 9943-9954, see especially the "Materials and Methods" section on oages 9944-9946.	1,3,6-9,11, 15-17

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ...... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers .... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a)..

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)